Integrated Master in Chemical Engineering

Biphasic Controlled Release of Active Pharmaceutical Ingredients from Biodegradable Implants

Master Thesis

Submitted by

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Abstract

The current work was carried out at *InnoCore Pharmaceuticals* and aimed at studying the production of implants by hot melt extrusion with controlled biphasic release as well the parameters influencing drug release kinetics.

Hot melt extrusion is a suitable process to produce implants with biphasic release kinetics as we were able to fulfill essential requirements such as size and good physical integrity. By changing the polymer from PDL 02 to PDL 05 an increase in the burst release was noticed while the presence of glycolide, a monomer that decreases polymer hydrophobicity, leads to a shorter lag phase. The state of the protein also influences the release kinetics, for non-stabilized protein the burst release is higher when compared with stabilized proteins. The implant diameter did not have an influence on the drug release profile.

The accumulation of acidic degradation products inside of the implant is crucial to obtain a bi-phasic drug release. For this study, the protein used denatured during release, the accumulation of degradation products inside of the implant leaded to an acidic micro-environment which is detrimental for the protein. At that low pH the protein loses the native conformation leading to a loss in the activity. Therefore, drugs which are highly sensitive to acidic environments seem not to be suitable for such therapeutic approach.

Resumo

O presente trabalho foi desenvolvido na *InnoCore Pharmaceuticals* e tinha como objectivo a produção de implantes através de extrusão com uma libertação bifásica de proteína assim como, estudar quais os parâmetros que influenciam a cinética de libertação do composto activo utilizado.

A extrusão é um método que permite a produção de implantes com libertação bifásica. A utilização do polímero PDL 05 em vez de PDL 02 faz com que exista um aumento no *burst release* enquanto que a presença de Ácido Glicólico, um monómero que diminui a hidrofobicidade do polímero, origina uma cinética de libertação com um menor período sem libertação de proteína. O uso de proteína estabilizada em inulina origina uma libertação com um menor *burst release*. Diferenças no diâmetro dos implantes não origina diferenças na cinética de libertação.

A acumulação de produtos de degradação do polímero no interior do implante é crucial para obter a libertação bifásica pretendida. Contudo, para princípios activos sensíveis a pH acídicos este não é um método de tratamento adequado uma vez que, a acumulação de compostos acídicos origina uma diminuição brusca de pH que faz com que a proteína perca a sua conformação nativa perdendo também atividade terapêutica.

Declaration

Declares, by her honor, that the current work is original and that all non-original contributions were duly cited with source identification.

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List of Abbreviations

API- Active pharmaceutical ingredient

NP – Nanoparticle

MP - Microparticle

PEG - Polyethylene glycol

HME – Hot-melt extrusion

NaOH – Sodium Hydroxide

HCl – Chloridric Acid

PES - polyester

UP – Ultra pure

PDL – Poly (D-Lactic acid)

PDLG – Poly (lactic-co-glycolic acid)

FD – Freeze-dried

SD – Spray-dried

SEM – Scanning electron microscopy

UPLC – Ultra performance liquid chromatography

IVR – *In vitro* release

TFA – Trifluoroacetic acid

N.A – Not applicable

ACN – Acetonitrile

EE- Encapsulation Efficiency

PBS – Phosphate Buffer Saline

1. Introduction

1.1. Background and Project Presentation

In the past few years the pharmaceutical industry developed a wide variety of new and more potent and specific drugs to treat different kinds of diseases. When a patient suffers from a chronic disease, usually repetitive dosages of medications for a long time have to be taken for treatment. Currently, alternative ways to deliver medicines are being created by developing controlled release drug loaded depot formulations. Controlled drug delivery formulations are usually based on polymeric micro and nanospheres, implants and pellets. These systems have been developed to address many difficulties associated with traditional drug administration methods. One of the major advantages offered by these systems is the capacity to protect sensitive bioactives such as proteins, which can easily be destroyed in the body because they are highly susceptible to changes of pH and, therefore, can easily lose their activity. Additionally, drug delivery systems can provide a constant drug level at the site of action, preventing peak-valley fluctuations and avoiding side effects. Finally, controlled drug delivery systems are usually designed in such a way that the number of administrations gets significantly reduced aiming at improving patient compliance.

In very specific therapeutic circumstances the standard progressive drug release from polymeric systems is not recommended. Instead, such circumstances require the release of the appropriate drug after a lag time and the same drug must not be released at all during the initial phase of dosage form administration which can be a challenge when designing an appropriate delivery system. Pulsatile release systems can be classified in multiple-pulse and single-pulse systems. These systems are frequently based on polymeric materials that release a drug almost instantly after the lag phase. Different delivery systems have been developed such as microchip-based devices to release a large number of drugs according to a preprogrammed time pattern. They have the ability to release a multitude of individual doses of one or even several substances from a multitude of drug reservoirs. Since each individual dose have a pulsatile release, combining the release profile of each individual dose can generate any desirable release profile. Although, this technology is limited in terms of controlling release kinetics and has some disadvantages such as the limited amount of drug that can be release from an individual reservoir and the lack of degradability in a biological system. Pulsatile delivery systems are usually based on polymeric materials that release a drug rather abruptly. The development of biodegradable implants seems to be a good approach to have a biodegradable, pulsatile drug delivery system that can offer alternative release timings according to rational modifications applied to the polymer composition.

A better understanding of the influence of the polymer, drug properties and processing parameters on the drug release profiles from implants can improve the formulation of an implant drug delivery system by inducing a desirable, controllable and predictable drug release profile.

Due to the confidentiality restrictions, the real name of the protein used cannot be disclosed. To preserve the identity of the protein of interest, which is hydrophilic and has around 45 kDa, from now it will be called protein X.

The objective of this work was to develop implants as a drug delivery system and study the effect of several process parameters such as drug loading, polymer composition and the state of the protein to be released from implants. One model protein was used and three states of that protein were analyzed: non-stabilized protein usually referred as coarse protein and stabilized protein using inulin prepared either by freeze drying or spray drying. The optimization of such therapeutic formulation aims at creating a bi-phasic release profile as represented in Figure 1. During an initial period a significant burst release should occur, followed by a lag phase where no protein is released and finally a second release period should be obtained where the rest of the bioactive molecule will be released from the implant. To achieve such desired release profile the characteristics of the polymers used will be crucial as hereafter discussed.

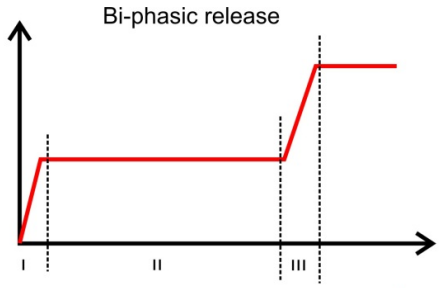

Figure 1. Schematic representation of a bi-phasic release kinetics.

1.2. Company presentation

InnoCore Pharmaceuticals is a privately owned biopharmaceutical company that specializes in the production of drug delivery devices. It was founded in 2003, based in Groningen in The Netherlands.

The core activities of InnoCore center on using a line of patented bioresorbable polymers named SynBiosys™ and SynBiosys Pro™ for drug delivery systems. These multi-block co-polymers are used for controlled release of small molecules, peptides and proteins. Drug delivery formulations developed by InnoCore include injectable solid implants, microspheres, drug eluting coating and injectable hydrogels. The focus of the activities performed lays mostly on R&D and pre-clinical trials, but InnoCore is gaining more experience on clinical trials as their products are increasingly used for commercial goals.

1.3. Work Contributes

The current project has helped InnoCore Pharmaceuticals to understand and characterize critical formulation parameters for the development of implants with a biphasic release profile. Problems associated to the effect of hot melt extrusion in the stability of proteins and the characteristics of polymers that can be applied successfully to the formulation process giving rise to a bi-phasic release will be addressed. Additionally, InnoCore Pharmaceuticals wants to understand whether their own platform of polymers (*SynBioSys*) offer advantages for such therapeutic application over standard PDLG and PDL polymers. By further understanding potential limitations on the process and potential competitive advantages, InnoCore Pharmaceuticals is going to get relevant technical information which can be crucial to decide about the future perspectives of the ongoing research about bi-phasic release from implants.

1.4. Thesis organization

The current chapter initiates the report of the project contextualizing and describing the motivation and main goals for the development of this work. Furthermore, Chapter 1 serves as a guideline to the overall work presented in the further chapters. In addition to Introduction, this work comprises six main chapters.

In Chapter 2 (State of the art) a short review of current achievements in the field is provided. The potential of drug delivery systems, the importance of polymers in drug delivery systems and the mechanisms of drug release from those devices are presented. The potential of hot melt extrusion for pharmaceutical applications is also discussed. The importance of protein stabilization in sugars as well the suitable sugars for that are examined.

In the section Materials and Methods (Chapter 3), a detailed description of the techniques is reported, including information about essential commercial products used. Materials and Methods are divided in 6 main sub-chapters. Fundamental techniques broadly used and/or representing key steps in the context of this work, have a short theoretical introduction previous to the detailed technical description.

Chapter 4 (Results and Discussion) gives the results and the main conclusions and probable indications that can be perceived from the results shown and possible interpretations for the results are explained.

Finally, Chapter 5 (Conclusions) gives an overview of the main findings, which reflect the novelty of the work presented. Additionally, future perspectives and recommendations are also discussed.

2. State of the Art

2.1. Drug Delivery Systems

Drug delivery systems may assume many alternative shapes and usually comprise the administration of an active pharmaceutical ingredient (API) to achieve better therapeutic effects or to reduce administration frequency. The latest advances and current challenges in drug delivery technology aim at providing the following benefits (1):

- Controlled delivery rate of the therapeutic agent;
- Maintenance of drug concentration in an optimal therapeutic range;
- Maximization of efficacy-dose relationship;
- Minimization of the needs for frequent dose intake;
- Enhancement of patient compliance.

Positive pharmacological properties of APIs may not be enough to trigger the desired biological effect as the molecule by itself needs to target and get internalized at the desired site of action. Thus, specific delivery mechanisms are often designed to promote controlled release and active targeting of encapsulated drugs, which ultimately improves therapeutic effects and also reduces potential toxic effects (2). In some cases, poor bioavailability, high toxicity, or simply the difficulty of targeting specific drugs to the desired body region make it impossible to use such a drug that otherwise would be considered a good therapeutic candidate. Therefore, the use of encapsulated drugs over traditional free drug administration may help overcoming these limitations (3). Furthermore, prolonged drug release would naturally give rise to less frequent dosing schedules which gets an increased importance in underdeveloped countries where poor patient compliance is often pointed out as major reason leading to treatment failure when fighting infectious diseases (4).

In order to promote a controlled delivery of bioactive agents, following rates and concentrations that maximize the therapeutic effect without exceeding limits of toxicity, specialists in the field of delivery systems have been testing an endless number of techniques and materials, such as metals and natural or synthetics polymers. The progress of biomaterials has been crucial for the advancement of drug delivery technology as they are the key elements who provide tunable release of both hydrophilic and hydrophobic drugs (5).

Although there are several parameters that may influence the final therapeutic outcome, the route of drug administration is possibly the most significant. Mechanisms of drug encapsulation and delivery have been designed in diverse shapes and formats, depending on the specificities of the API and disease in consideration (2). For instance, nanoparticles (NPs) due to their small size and ability to cross biological barriers are suitable for systemic administration (6). On the other hand, bigger particles in the range of 1-5 μm (microparticles

– MPs), owing to their higher density are often considered the ideal option for pulmonary drug delivery as they easily get deposited at the deep lung (7). Alternatively, implants have been considered for prolonged subcutaneous drug release (8).

2.2 Polymers in Drug Delivery Systems

Biodegradable polymers are receiving increasing attention for their use in a wide variety of medical and pharmaceutical applications. The use of synthetic polymer-based materials may provide considerable improvements in medical applications due to their thermal and mechanical properties and their natural decomposition into non-toxic products that are easily metabolized or excreted by the human body (9). The use of aliphatic polyesters have a leading position since hydrolytic and/or enzymatic chain cleavage yields hydroxy carboxylic acids, which in most cases are ultimately metabolized (10). The most common synthetic biodegradable polymers used in medical applications are polylactide, polyglycolide, and poly(ε-caprolactone). Final polymer key properties, namely degradation rate, tensile properties and surface chemical composition, can be optimized by copolymerization or blending of homo- and/or copolymers.

2.2.1. SynBiosys Polymers

InnoCore Pharmaceuticals owns a patented polymer platform called Synbiosys, which consists of biodegradable multiblock copolymers (MBCPs) prepared by chain-extension of pre-polymers that incorporate D,L-lactide (L), glycolide (G), polyethylene glycol (PEG) (P) and **ε** -caprolactone (C). By varying the length and monomer content of the building blocks several properties can be adjusted, such as glass transition temperature, hydrophobicity, swelling ratio, permeability and degradation rate.

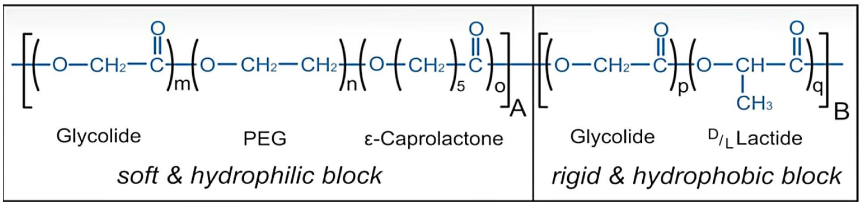

Figure 2. Schematic representation of the molecular structure of a SynBiosys polymer.

The main attributes of SynBiosys polymers are present in Table 1.

Table 1. Key attributes of SynBiosys multi-block polymers.

Precise delivery of small molecules, peptides and proteins
Controllable release profiles (from days to months)
Drug loading of 1 to 50 wt%
Biocompatible and regulatory approved building blocks
Completely bioresorbable with adjustable degradation rates
Drug integrity and bioactivity preserved
Low temperature processibility
Patent protected

SynBiosys multiblock copolymers present improved characteristics when compared to commonly used biodegradable polymers, namely improved thermal, mechanical and processing characteristics. When a multiblock copolymer is composed of lactide, glycolide and/or **ε**-caprolactone segments, the polymer absorbs small amounts of water and, consequently, is characterized as non-swellable polymer. On the other hand, when PEG is introduced in one of the pre-polymers, it becomes possible to obtain polymers with higher hydrophilicity since they would be composed of a water swellable segment combined with other more hydrophobic, non-swellable segment. Playing with the composition and content of both hydrophilic and hydrophobic segments the polymer swelling degree and degradation rate can be controlled, being possible to manipulate drug release kinetics. This control over the copolymer properties is the most interesting characteristic of the SynBiosys platform.

2.2.2. Polymer degradation

The polymer chains can be degraded by two principal ways: chemical hydrolysis and enzymatic reaction. Many of the synthetic polyesters used in medical or pharmaceutical applications degrade mainly by pure hydrolysis, thus, polymer swelling degree due to water absorption provides a major influence in terms of degradation kinetics (11).

The rate of degradation of a polymer may additionally be influenced by several other factors like the type of chemical bond in the polymer back-bone, hydrophobicity, molecular weight, crystallinity, copolymer composition and the presence of low molecular weight compounds (12). During the process of polymeric degradation, the loss of mechanical stability should be carefully analyzed since when it occurs too fast high concentrations of degradation products may accumulate leading to undesirable local toxicity (13). As any biodegradable polymer

contains hydrolysable bonds, the specific degradation rate will always depend on the type and stability of chemical bond between monomers. For example, polymers containing anhydride or ortho-ester bonds are the most reactive ones with a faster rate of degradation. The ester bonds will degrade slower and carbonates offer good resistance to hydrolysis (13).

The hydrolysis of the polymer bonds produces monomers and oligomers. Such degradation products, if small enough, will diffuse to the outside of the polymer block once the cleavage process has ended – a mechanism usually described as polymer erosion, which consists on the sequential loss of mass from degradable polymer matrices. Figure 3 shows the general mechanism of polymer erosion. In brief, the polymer chains will first degrade into monomers and oligomers and then they will dissolve and diffuse into the environment (14).

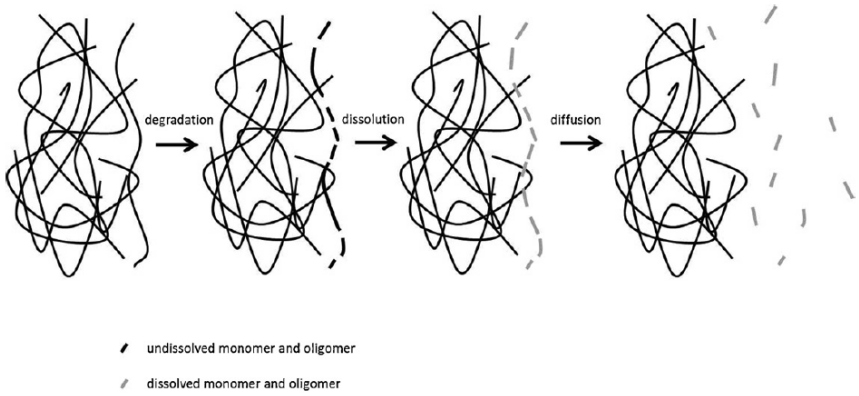

Figure 3. Schematic representation of the mechanism of polymer erosion (14).

In general terms, polymeric devices can degrade via one of two alternative mechanisms (15). They can either degrade via bulk erosion or surface erosion. This last consists on an outside-in degradation process, where the environment will initially affect the surface until the polymer is completely degraded. Bulk erosion and degradation occurs when the polymer device degrades homogeneously over time as represented in Figure 4. If the water uptake by the polymer matrix is faster than polymer degradation, bulk degradation takes place due to uniform hydrolysis. However, several studies show a heterogeneous degradation mechanism. The degradation products generated inside of the polymeric device autocatalytically accelerate the degradation process due to an increase in the amount of carboxylic acid end groups which are responsible for the faster degradation in the center of the device when comparing to the surface (16). On the other hand, if degradation is faster than the water ingress, hydrolysis will only occur at the edges and the polymer will be subjected to surface erosion (15). The degradation mechanism is usually influenced by the hydrophobicity of the polymer as a hydrophobic polymer matrix will probably cause surface degradation and a more hydrophilic polymer matrix will probably be faster degraded via bulk erosion (12).

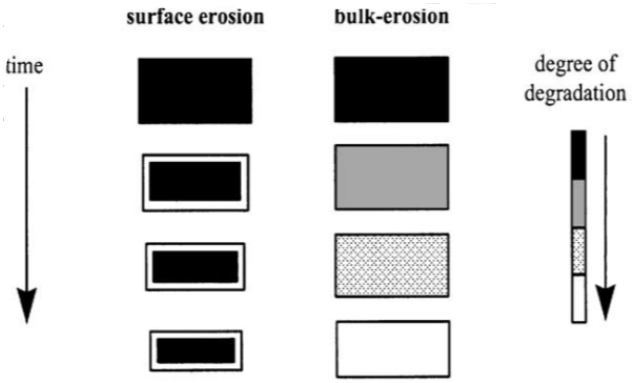

Figure 4. Schematic representation of surface erosion *versus* bulk erosion (15).

In addition, bulk and surface degradation differ in terms of rate profile. Ideally, bulk degradation has a linear degradation curve due to the homogenous degradation and surface degradation has a logarithmic degradation curve due to a decreasing surface area over time. Most models describe a single mechanism to produce quantitative predictions on degradation, but these models represent an idealization of reality. In practice, both mechanisms occur at the same time (15).

2.2.3. Mechanisms of Drug Release from Polymeric Delivery Systems

In order to develop an efficient controlled-release system for therapeutic drugs is important to understand the release mechanism and the physico-chemical processes that mostly influence the release kinetics. The mechanism of release is the rate limiting step or series of rate limiting steps that control the rate of drug release from a device until release is exhausted. The major release mechanisms include: diffusion, solvent penetration/device swelling, degradation and erosion of the polymer matrix or a combination of these mechanisms occurring on different time scales that leads to a more complex release process (17). In Figure 5 there are presented schematic representations of these individual release mechanisms. Many processes can influence the rate of drug diffusion and degradation kinetics, for example, polymer-drug interactions, drug-drug interactions, water absorption and pore closure. By knowing and understanding such processes we can easily control drug release mechanisms.

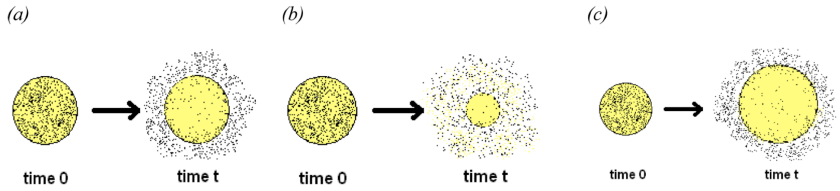

Figure 5. Schematic of common release mechanisms (a) Diffusion (b) Polymer degradation and erosion and (c) solvent penetration and device swelling (17).

Transport through the surface of the polymer usually happens when a drug loaded depot formulation comes in contact with the medium (aqueous solution) as drug on the surface of

the polymeric matrix can easily diffuse to the release medium (17). This mechanism is also called burst release, which is characterized, with a rapid release of API from the polymeric matrix in a short period of time. Diffusion is the random movements of molecules from an area of high concentration to an area of lower concentration. As exposure of the drug loaded depot formulations with release medium proceeds, drug is released progressively from the polymeric matrix to the release medium. The release of drug from the matrix could be slow (17). This might come due to the polymer which could be dense or that the "cracks" in the polymeric matrix are not big enough in order for the API to diffuse through it. The polymeric matrix degrades when its exposure to medium proceeds. The water (from the medium) hydrolyzes the polymeric matrix into soluble byproducts (i.e. oligomers and monomers) and such elements can accumulate in the center of the matrix. During this phase, the polymeric matrix falls apart leading to a fast release of the drug molecules which are originated from the center of the polymeric matrix (17).

In the case of a biphasic release behavior as presented in Figure 6, the release during the phase I is related to an immediate burst release, when drug molecules close to the surface in contact with the aqueous medium diffuse for the outside of the device (Figure 7). After this fast drug release period, the release occurs slowly and during this lag phase (II) the device starts slowly degrading. During this phase the matrix is still dense and the pores formed by the diffusion of the drug are not big enough to release the API. Due to the accumulation of degradation products inside of the polymeric device the pH a decrease leading to an increase in the degradation rate due to the autocatalytic effect of the degradation products and at a certain point the device disintegrates releasing the remaining loaded drug. Such moment can be manipulated by engineering the polymeric materials in use.

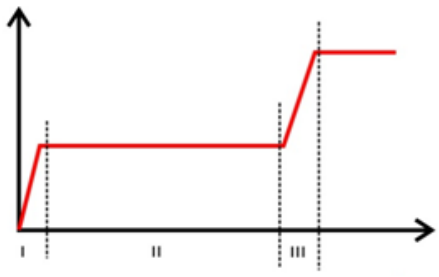

Figure 6. Schematic representation of a biphasic drug release kinetics.

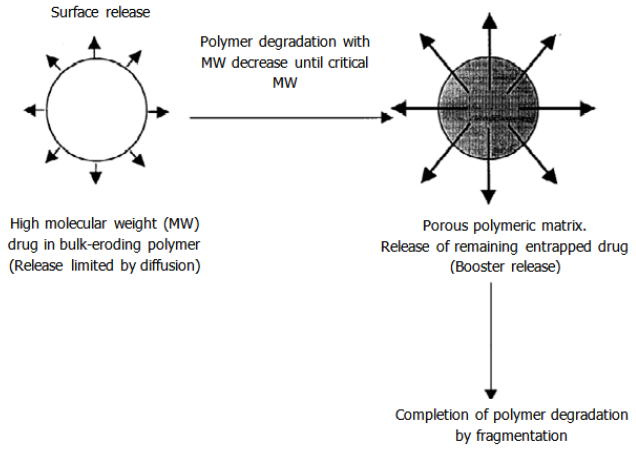

Figure 7. Theoretical bi-phasic release of a drug from a bulk-eroding polymeric system (Adapted from (18).

2.3. Hot-melt extrusion

Hot melt extrusion (HME) technique was first introduced in the plastics industry in the mid-nineteenth century to manufacture pipes (19). Since then, it has mainly been used in plastic, rubber and food manufacturing industry (20). Currently more than half of all plastic products on the market including, bags, sheets and pipes are manufactured via HME. Thus, various polymers have already been used to form different shapes for a variety of domestic and industrial applications (20). This technology has proven to be a robust method for producing numerous drug delivery systems including granules, pellets, sustained release capsules and implants (19), and therefore it has also been found to be useful in the pharmaceutical industry (20). Hot melt extrusion can be globally defined as the process of converting a raw material into a finished product by forcing them through a die applying heat at the same time (21).

Hot melt extrusion can be classified into two categories: ram extrusion and screw extrusion. Ram extrusion is an extrusion with a simple design and with a discontinuous operation (19). The process occurs with the positive displacement ram which generates high pressure to push materials through the die – process represented in Figure 8. Materials are heated in the cylinder until molten materials are obtained and subsequentially the material passes through a die to obtain the desired shape (19). The ram extrusion has limited melting capacity and poor temperature uniformity. The products obtained by ram extrusion have poor homogeneity when compared with products obtained by screw extrusion (19).

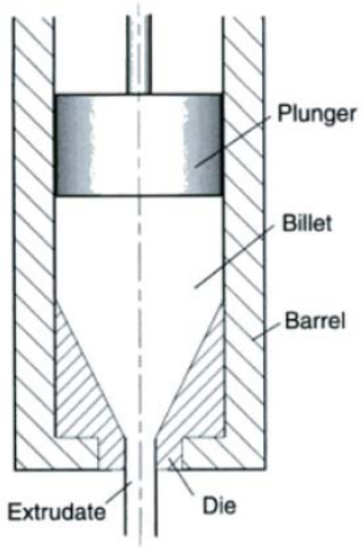

Figure 8. Schematic representation of a ram extruder (22)

A screw extruder consists in three sections: feeding zone, transition zone and metering zone which is represented in Figure 9 A starting material is feed into the feeding zone. The material is prepared to be conveyed along the barrel (23). A solid plug from the feeding zone is transferred to the transition zone where it is molten, mixed and compressed due to friction between the mixture, barrel and rotating screw (23). The driving force for the extruded material is the friction on the surface of barrel (23). During the process is necessary to apply heat that can be supplied by two sources: shearing of the rotating screw and electrical heating (19). The material reaches the metering zone in the form of a molten uniformly mixture, this zone acts like a flow reducer and a controller of the outcome rate (23).

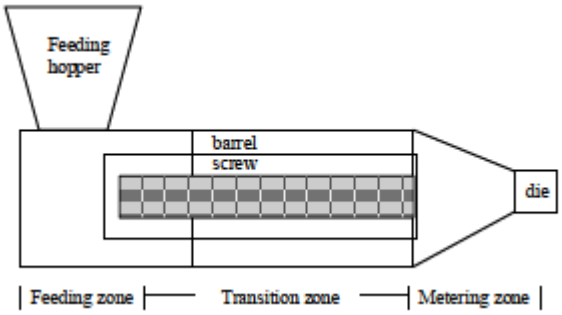

Figure 9. Schematic representation of screw hot melt extruder in a horizontal view (22)

The screw extruders can be classified as single screw and twin screw extruders (20). The single screw extruder is a simple equipment formed by a screw rotating inside a barrel which is used for solid transportation, melting, mixing and pumping (19). A barrel convers the screw and contains three or more heating zones to elevate temperatures at the screw and barrel until meeting the desirable temperature. Pressure inside the barrel is generated by melting and mixing viscous materials and pumping them through a die (19). Twin screw extruders consist in two screws inside a barrel side by side (20). The use of two screws makes the

equipment more versatile once the direction of screw rotation can be changed (22). The two screws can either rotate in the same direction or can rotate in different directions (19).

The transport mechanism and the mixing ability are the main differences between the single screw extruder and twin extruder (22). In a single screw extruder the transport mechanism is based on frictional forces in the solid conveying zone and viscous forces in the melt conveying zone. When compared with twin screw extruder the single screw extruder exhibits poor mixing capacity. In a twin screw extruder the mixing occurs both at a macroscopic level, where the material is exchanged from one screw to another, as well as at the microscopic level, where the mixing occurs at the high-shear regions of screw elements interactions (22).

The use of twin screw extruder offers several advantages when compared to single screw extruder once in the first one the residence times are shorter, the process of melting is more stable and to achieve the equivalent output smaller equipment is required (22).

When compared with conventional methods, hot melt extrusion offers several benefits, some of them are represented in Table 2.

Table 2. Advantages of hot-melt extrusion (Adapted from (24).

Feature	Benefit
Solvents not required	Environmentally friendly, economical; No residual solvent in final product
Continuous process	Fewer unit batches required; Efficient scale-up
Intense mixing and agitation achieved	Improved content uniformity
Compressibility not required	Useful for powders with low compressibility index
Polymers serve multiple purposes	Less number of excipients required; Cost effective
Greater thermodynamic stability than that produced by other hot-melt methods	Less tendency towards recrystallization

The extrusion is a continuous method and the extruders can be used for mixing, melting and extrusion or reacting materials and this can be processed in a single operation unit (22). It is an anhydrous and solvent less process, which avoids the hydrolysis of the materials being used and also decreases the toxicity of the final product once there is no remaining toxic solvent (22).

2.4. Protein Stabilization

The use of proteins as pharmaceutical drugs is becoming increasingly important to treat several diseases and disorders (25). Usually proteins are obtained in aqueous solution, however, many of them are unstable in aqueous medium which reduces their potential shelf life (25). These problems can be easily overcome by drying the protein however, during the drying processes, e.g. freeze drying, spray drying, the protein is subjected to harsh conditions through which its activity can be severely reduced. Thus, it's necessary to add protective agents to prevent the adverse and sometimes irreversible effects of drying processes (25).

Sugars can be used as protective agents for proteins during drying and storage. During drying, the water molecules surrounding the proteins are gradually replaced by sugar molecules. Being a polyol, the sugar molecules form multiple external hydrogen bonds with the protein by which structural integrity of the protein is maintained (25). The crystallization of the sugar should be low once the crystallization is accompanied by phase separation through which the interaction of the sugar molecules with the protein and thereby the protection is lost (25). It is essential that the sugar remains in the glassy state during handling and storage (*Tg* above room temperature) since the molecular mobility in this state is extremely low which makes the protein immobilized in the glass. This immobilization also protects the protein against degenerating effects after the drying process. The restricted molecular mobility disables some crystallization of the sugar for long periods of time. On the other hand, at temperatures above *Tg*, the mobility increases and the sugar protection is partially lost (25). The increase in the molecular mobility caused by higher temperatures also induces rapid crystallization of the sugar (25).

The *Tg* of a sugar glass depends on the nature of the sugar and on the water content. Water acts as a plasticizer and the uptake of water by the sugar results in a strong decrease of the *Tg* (25).

Sugars must be non-reducing agents since the reducing groups can react with amine groups of the protein and such reactions, which are the first of a cascade of reactions – *Maillard* reaction, can severely affect the protein activity (25).

When freeze drying is used as a drying process it is preferable to use sugars with high *Tg* because the sample temperature should remain below the *Tg*. When the temperature is above the *Tg*, the freeze concentrated fraction is in the liquid or rubbery state thus the mobility is high. Furthermore, the protein concentration in the freeze drying fraction is high, therefore the degradation rate will increase when compared to the starting solution. Moreover, the sugar concentration is very high and crystallization can easily occur.

Additionally, freeze drying above the *Tg* results in a collapsed cake while below the *Tg* results in a porous cake. The last one is preferred because it can be easily reconstituted or processed (25).

The oligosaccharide inulin is a potent lyoprotectant, since it holds physiochemical properties which are essential for any stabilizing agent, such as high *Tg*, low number of reducing groups and low crystallization rate. In the literature inulin sugars have already been successfully used for the stabilization of proteins (26).

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals used

Table 3. Chemicals used in the different experiments and solutions preparations.

Chemical	Purity/Type	Supplier	MS#
Acetonitrile	Ultra LC-MS	Actu-All chemicals	423
Dichloromethane	99.5% purity, stabilized with ethanol	Acros organics	131
Dioxane	99% purity, stabilized	Acros organics	422
Di-sodium Hydrogen orthophosphate	anhydrous	Fisher Scientific	169
Potassium hydrogen orthophosphate	>99% purity	Fisher Scientific	168
Sodium Azide	99% purity	Acros organics	282
Sodium Chloride	>99% purity	Merck	166
Potassium Chloride	>99% purity	Fisher Scientific	167
Protein X		RuG	N.A
Stabilized Protein X	9%	RuG	N.A
InX	Spray-dried, protein X:Inulin ratio 1:10	RuG	N.A
Trifluoroacetic acid		Millipore	159
Ultra-pure water	RO, Ultra filtrations, UV, 0,22 µm filtered, resistivity = 18,2 MΩ.cm at 25°C	Innocore	N.A

3.1.2. Equipment used

Table 4. Equipment used for all the experiments and solutions preparation.

Equipment	Supplier/Type	INN# / MS#
2-decimal balance	Kern	2010/015, 2009/027
4-decimal balance	Mettler Toledo	2011/025
5-decimal balance	Mettler Toledo	2013/021, 2011/006
Centrifuge	Thermo Scientific	2013/002
Conveyer belt	Thermo scientific	2009/038
Digital thermoregulator	VTF	2010/012
Extruder	HAAKE MiniLabII, Thermo scientific	2008/023
Freeze-drier	Zirbus technology, Sublimator 2x3x3/5	2009/007
Heating magnetic stirrer	VELP SCIENTIFICA, Arex.X	2011/011
Manifold	Millipore	2011/035
Oven (37 C)	Hermeus	2004/007
SEM	JEOL, Neoscope	2011/039/A
pH apparatus	inoLab	2012/025
UPLC	Waters, Acquity	2010/002
UPLC column	Acquity C_4 BEH peptide separation column ; 1.7 µm, 2.1x50mm ; Waters	N.A
Vacuum pump	Millipore	2011/036
Vacuum oven	Fistreem	2011/028

3.1.3. Disposable materials used

Table 5. Disposables used during the experiments.

Disposable	Supplier/Type	MS#
Membrane filters	Millipore, Durapore, 0.45 µm	347
UPLC plate	Millipore	373

3.1.4. Polymers used

Table 6. Polymers used in the implant formulations.

Composition	Batch#	IV (dL/g)	*Tg* (˚C)	Tm (˚C)
30CP15C20-C40	RCP-1206	0.76	-60.1	51.7
Poly (D,L Lactide)	Purasorb® PDL 02	0.20	34-39	-
Poly (D,L Lactide)	Purasorb® PDL 05	0.50	42-47	-
Poly (D,L Lactide-glycolide) (50/50)	Purasorb® PDLG 5002	0.20	32-37	-
Poly (D,L Lactide-glycolide) (50/50) (Acid terminated)	Purasorb® PDLG 5002A	0.20	40-45	-

3.2. Solutions and buffers

3.2.1. Phosphate buffer (100mM, pH 7.4)

The components present in Table 7 were quantitatively added to 900 mL of UP water and dissolved using a stirring bar. After the components were dissolved, the solution pH was measured and adjusted when necessary with 1 M NaOH or 1M HCl until a pH of 7.4 ± 0.05 was reached. The stirring bar was then removed and UP water was added in order to have a final volume of 1000 mL. After measuring the pH and adjust it to 7.4 ± 0.05, the buffer was filtrated with a 0.2 µm polyester (PES) filter.

Table 7. Amount of the different chemical products added to the UP water for the Phosphate buffer (100mM,pH 7.4) preparation.

Chemical	Amount
KH_2PO_4	2.48 g ± 0.05 g
Na_2HPO_4	11.61 g ± 0.1 g
NaN_3	0.20 g ± 0.05 g
1 M NaOH	For pH adjustment
1 M HCl	For pH adjustment
UP water	Add to 1 L

3.2.2. Phosphate buffer (5mM, pH 7.0)

The components present in Table 8 were quantitatively added to 900 mL of UP water and dissolved using a stirring bar. After the components were dissolved, the solution pH was measured and adjusted when necessary with 1M HCl until a pH of 7.0 ± 0.05 was reached. The stirring bar was then removed and UP water was added in order to have a final volume of 1000 mL.

Table 8. Amount of the different chemical products added to the UP water for the Phosphate buffer (5mM,pH 7.0) preparation.

Chemical	Amount
Na_2HPO_4	0.514 g ± 0.05 g
NaH_2PO_4	0.330 g ± 0.05 g
1 M HCl	For pH adjustment
UP water	Add to 1 L

3.3.3. Phosphate Buffer Saline (10mM,pH 7.40)

The components present in Table 9 were quantitatively added to 900 mL of UP water and dissolved using a stirring bar. After the components were dissolved, the solution pH was measured and adjusted when necessary with 1M HCl until a pH of 7.4 ± 0.05 was reached.

The stirring bar was then removed and UP water was added in order to have a final volume of 1000 mL.

Table 9. Amount of the different chemical products added to the UP water for the Phosphate buffer (10mM,pH 7.0) preparation.

Chemical	Amount
NaCl	8.00 g ± 0.10 g
KCl	0.20 g ± 0.05 g
KH_2PO_4	0.24 g ± 0.05 g
Na_2HPO_4	1.15 g ± 0.05 g
NaN_3	0.20 g ± 0.05 g

3.3. Preparation of freeze-dried inX

To obtain an aqueous inulin solution of 50 mg/mL, 6.847 g of inulin was dissolved in 138 mL of 5mM phosphate buffer solution (pH=7.0) under continuous heating in a hot plate (80°C) in a container closed with parafilm. After complete dissolution of inulin, it was allowed to cool down to room temperature (20°C). Finally, 568 mg of protein X was gently dissolved in 110 mL of the inulin solution resulting in a protein concentration of 5 mg/mL.

The solution of inulin and protein X was frozen and freeze dried (Zirbus technology, Sublimator 2x3x3/5) overnight according to the procedure described in Table 10.

Table 10. Freeze-drying program.

Step	Phase	Time (min)	Temperature (°C)	Vacuum (mbar)
01	Freeze	10	-45	--
02	Freeze	60	-45	--
03	Dry	60	-45	30
04	Dry	240	-5	0.220
05	Dry	480	-5	0.220
06	Dry	120	10	0.055
07	Dry	60	10	0.055
08	Dry	60	25	0.055
09	Dry	20	25	0.055
10	Dry	Until removal of the sample	20	0.001

3.4. Implants formulation

3.4.1. Biphasic release implants prepared by Hot-melt extrusion

Hot melt extrusion was performed using a HAAKE Mini Lab II from Thermo Scientific co-rotating twin-screw extruder equipped with conveyer belt to stretch the molten material. The extrusion was performed with a fixed screw speed of 10-15 rpm.

The powder blend was prepared manually by mixing the core mix with the polymer in different ratios. The core mixture was composed by protein X, inulin and mannitol. In Table 11 is presented the extent of the experiments performed during this work where the influence of several variables has been evaluated.

For the PDL 02 implants, the polymer was grinded in a mortar and then sieved in order to collect the fraction with particle sizes below 180 μm. For the other materials, the grinding step was performed with a kitchen grinder and dry ice. The final material was dried on the vacuum oven overnight and then sieved to collect the fraction with particle sizes below 180 μm. The selected polymer was then mixed with the core mixture in a mortar for approximately 10 minutes. Afterwards, the mixture of polymer and protein was fed to the extruder and the implants with different diameters (0.6, 1.0 and 1.4 mm) were finally collected.

A cylindrical die of 1.5 mm was used to modify the diameter of the implants by changing the speed of the conveyer belt. The extruded samples were stored in a freezer at -20^{o}C until further analysis.

In order to understand the biphasic release kinetics from extruded implants several parameters need to be analyzed. Implant diameter, ratio between core material and polymer, presence of mannitol, particle size of protein used and polymer composition were all considered the critical formulation parameters.

Table 11. Design of experiments

Experiment	Polymer	Ratio core mix:polymer	API	Core mix composition (API : inulin :mannitol)	Temperature (°C)
JA1408	PDL 02	1:10	Protein X	1:10:14	70
JA1410	PDL 05	1:10	Protein X	1:10:14	75
JA1416-01	PDL 02	1:10	inX (SD)	1:10:14	70
JA1416-02	PDL 02	1:10	inX (SD)	1:10:0	70
JA1424-01	PDL 02	1:10	inX (FD)	1:10:0	70
JA1424-02	PDL 02	2:10	inX(FD)	1:10:0	70
JA1430-02	PDL 02	3:10	inX(FD)	1:10:0	70
JA1434	PDLG 5002	1:10	inX (SD)	1:10:0	50
JA1452	30CP15C20-C40-7	1:10	inX (FD)	1:10:10	50

3.5. Implants characterization

3.5.1. Surface morphology

Scanning electron microscopy (SEM) is based on the incidence of a beam of accelerated electrons on the sample. These accelerated electrons interact with the sample, exciting its atoms which emit secondary electrons. According to the angle between the primary beam and the surface of the sample, it is possible to detect and analyze the surface topography.

In order to evaluate the surface morphology of the developed implants, SEM was performed using a *Neoscope, Jeol*. The sample was first placed in the carbon conductive tape and subsequently coated with a thin gold layer (3 minutes coating time). The sample was then introduced in the microscope, vacuum was applied and the implants were imaged using a 10 kV electron beam.

3.6. *In Vitro* release study

For the In vitro release (IVR) study, approximately 40 mg of each implant was transferred to a 2mL plastic tube (N=3). Then, 2 mL of release buffer (100 mM Phosphate buffer, pH 7.4) was added to the same tube, which was closed with the screw cap and placed in the oven at 37°C.

Samples were taken at the beginning of the IVR study and after 3h and 1, 2, 3, 4, 7, 14, 21, 28 days (or until complete release was observed). Afterwards, 2 x 0.9 ml of the supernatant was transferred to sample tubes: one to analyze by Ultra performance liquid chromatography (UPLC) and other to keep in the freezer. In order to refresh the release buffer and restore the original volume, 1.8 ml of fresh buffer was added to the original plastic tube.

3.7. Quantification of protein X by Ultra Performance Liquid Chromatography

Ultra Performance Liquid Chromatography (UPLC) is a chromatographic technique that allows the identification and quantification of chemical compounds. This technique was used to determine the amount of protein X released from the implants. Table **12** presents the UPLC method to measure protein X released from the implants.

Table 12. UPLC method to measure Protein X

Eluent	A : 0.025% Trifluoroacetic Acid (TFA) in water B : 0.025% TFA in ACN
Flow	0.7 mL/min
Injection volume	10 µL
Number of injections	2
Sample temperature	4°C
Run time	5 min
Gradient	0 min : 30% B 0-3 min : 30% to 60% B 3-3.01 min : 60% to 90% B 3.01 to 4 min : 90% to 30% B
Column	Acquity C_4 BEH peptide separation column ; 1.7 µm, 2.1x50mm ; Waters
Column temperature	50°C
Detection	UV, 213 nm

The UPLC analysis was performed using 200 µL of sample in a 96-well plate. Samples and standards were filtrated through a 96 well filter plate with 0.2 µm pore PVDF filter prior to measurement. For the standard preparation, protein X (coarse) was used. First, a stock solution with a concentration of 1000 µg/g was prepared by dissolving 20 mg of protein X in 20 g of 10mM PBS, pH 7.4. The stock solution was diluted to desirable concentrations (5 -250

μg/g), as listed in Table **13**. Standards were labeled and stored at 4°C for a maximum period of 1 month.

Table 13. Dilutions used to prepare protein X standard solutions from a 1000 μg/g stock solution.

Standard	Amount protein X (g)	Amount Buffer (g)	Concentration (μg/g)
Stock	0.020	20.00	1000.00
	Amount Stock (g)	**Amount Buffer (g)**	
1	1.00	3.00	250.00
2	0.80	3.20	200.00
3	0.60	3.40	150.00
4	1.30	11.70	100.00
	Amount Std 4 (g)	**Amount Buffer (g)**	
5	3.00	1.00	75.00
6	2.00	2.00	50.00
7	1.00	3.00	25.00
8	1.30	11.70	10.00
	Amount Std 8 (g)	**Amount Buffer (g)**	
9	2.00	2.00	5.00
10	0.00	4.00	0.00

Low concentrations are also measured in order to determine the Limit of detection (LOD) and the Limit of quantification (LOQ) of protein X by UPLC. The determination of these values is important in order to know the lowest concentration of protein that could be detected and differentiate from a blank by the UPLC (LOD) and the lowest concentration of protein that could be reliably quantify by UPLC (LOQ). To do this, 7 standards from 0 to 12 μg/g of protein X are measured via UPLC. The LOD and LOQ are calculated via regression analysis, and by using the formulas below.

$$\boldsymbol{Standard\ deviation: SD = \sqrt{\frac{\sum(y-y')^2}{n-2}}} \qquad \textbf{Equation 1}$$

$$\boldsymbol{LOD = \frac{3.3*SD}{Slope}} \qquad \textbf{Equation 2}$$

$$\boldsymbol{LOQ = \frac{10*SD}{Slope}} \qquad \textbf{Equation 3}$$

The slope is determined via regression analysis of the validation line. The variables y and y' correspond to the experimental response and the calculated response of the samples and n corresponds to the number of points measured.

3.8. Content determination

For the characterization of the implants, a content determination method is necessary in order to determine the real loading of protein X.

For content determination study 80 mg of implants were dissolved in 5 mL of ACN in order to dissolve the polymer and then extract the drug from the polymeric device. After complete dissolution of the polymer, the solution was centrifuged at 12000 rpm for 10 min. 4.8 mL of supernatant was removed and 4.8 mL of 100mM Phosphate buffer, pH 7.4 was added in order to dissolve the API. After completely dissolution of the protein in the buffer the samples were analyzed by UPLC.

4. Results and Discussion

4.1. Detection and Quantification of Protein X by Ultra Performance Liquid Chromatography

The UPLC method described in the previous section leads to a chromatogram identical to the one presented in Figure 10.

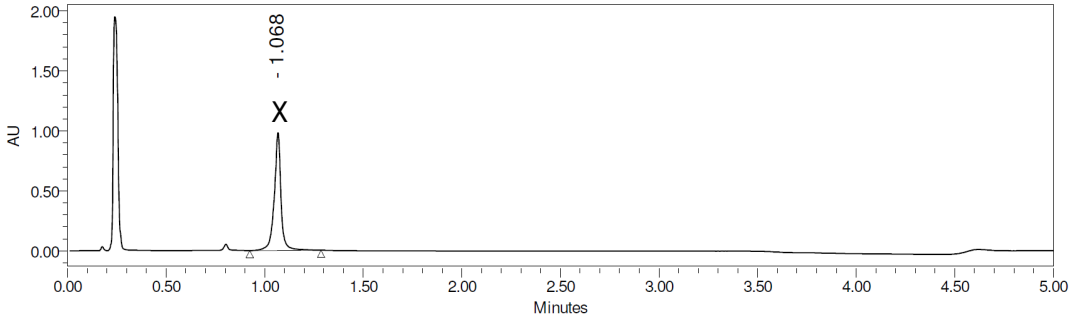

Figure 10. Chromatogram obtained by the UPLC method described in the Materials and Methods section.

From the chromatogram represented in Figure 10 it is possible to observe that the protein X peak is clear and separated from the other peaks. This makes possible to quantify the area of this peak and to obtain a calibration line, which allows the creation of a direct relation between protein concentration and peak area.

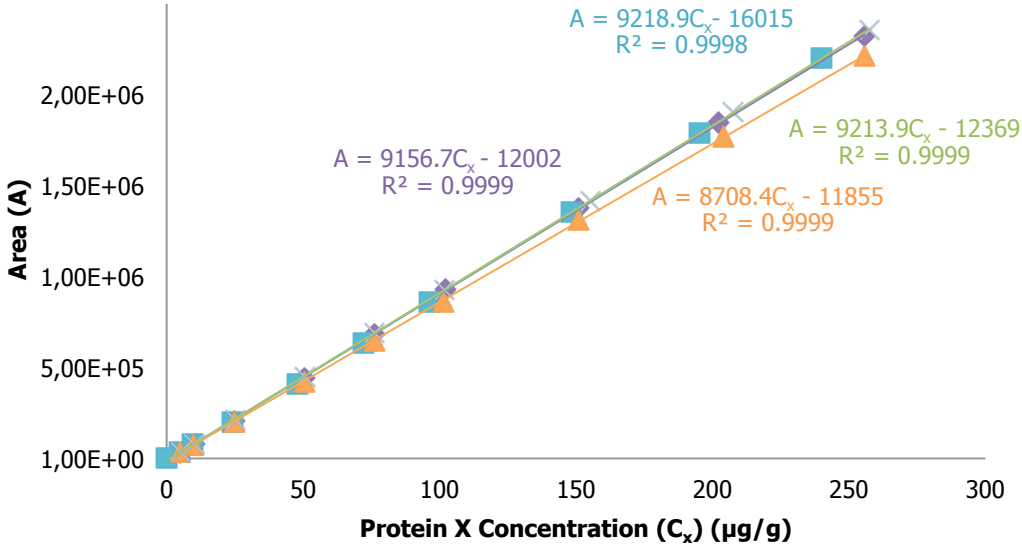

Figure 11. Schematic representation of calibration lines used for UPLC measurements.

In Figure 11 several calibration curves used for the protein X quantification are represented. These calibration lines present a high linearity and low deviation between different calibration series, which indicates that the UPLC method is reproducible. The limit of detection (LOD) and the limit of quantification (LOQ) for the method were estimated and the results are present in Table 14.

Table 14. Limit of quantification and limit of detection of protein X via UPLC

LOD	0.4 µg/g
LOQ	1.2 µg/g

The protein X presence on samples could be detected by the UPLC starting from a concentration of 0.4 µg/g and its quantification is reliable starting from 1.2 µg/g.

4.2. Surface morphology analysis of the implants

4.2.1. Effect of the material

Figure 12 shows the differences in the surface morphology of implants made with different polymers, when all the remaining characteristics were unchanged. The surface of the implant prepared with PDL 02 is rougher and implants made with PDL 05 present some flow lines on the surface. PDL 05 has a high molecular weight thus, presents a higher melt viscosity and flows less when compared with formulations with PDL 02. This higher melt viscosity leads to the formation of lines on the surface when the melt flow exits the die.

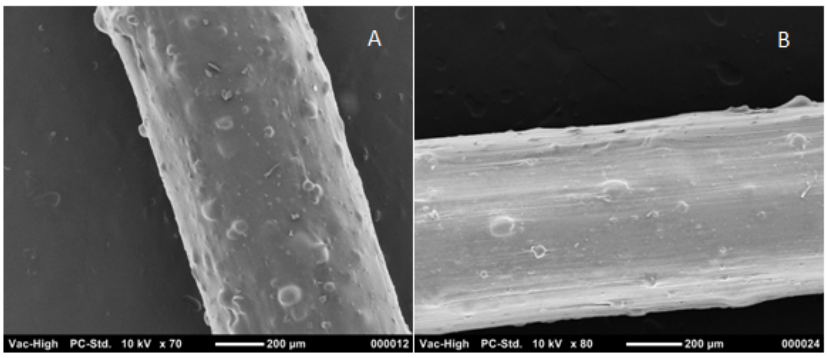

Figure 12. Scanning electron micrographs of implants made of (A) PDL02 (JA1408) and (B) PDL05 (JA1410).

4.2.2. Effect of the protein particle size

Protein stabilization via freeze-drying leads to bigger particle sizes when comparing to the ones obtained from stabilization via spray drying. Both SEM pictures present in Figure 13 were prepared with the same drug loading and without mannitol. In Figure 13 (A) is represented a formulation with spray-dried protein X while in Figure 13 (B) is an implant loaded with freeze-dried protein X. The formulation prepared with freeze-dried protein offers a rougher surface when compared with the same formulation with spray-dried protein. For this situation the protein particles are dispersed in the viscous polymeric flow, thus, if the particles are big a rough surface will be formed.

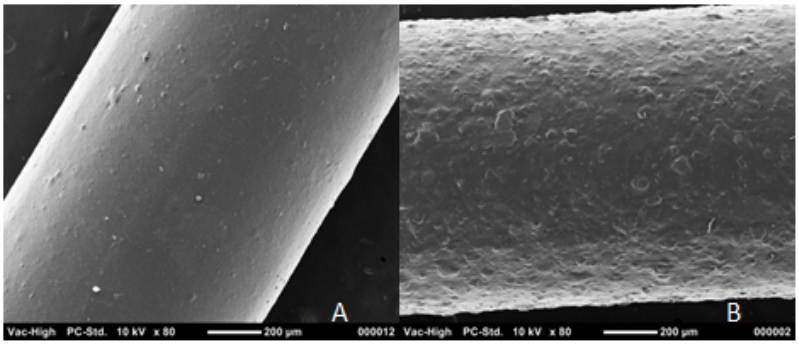

Figure 13. Scanning electron micrographs of implants made with (A) Spray-dried (JA1416-02) and (B) Freeze dried (JA1424-01) stabilized protein.

4.2.3. Effect of the drug loading

All formulations present in Figure 14 were made with PDL02 and with a core mixture with freeze-dried stabilized protein. The increase of drug loading leads to rougher implants. During extrusion the protein remains in the solid state dispersed in the viscous polymeric flow. However, as the material leaves the extruder, polymer tends to solidify and the solid particles remain in the matrix. The ones closer to the surface are finally responsible for the visual roughness of the implant.

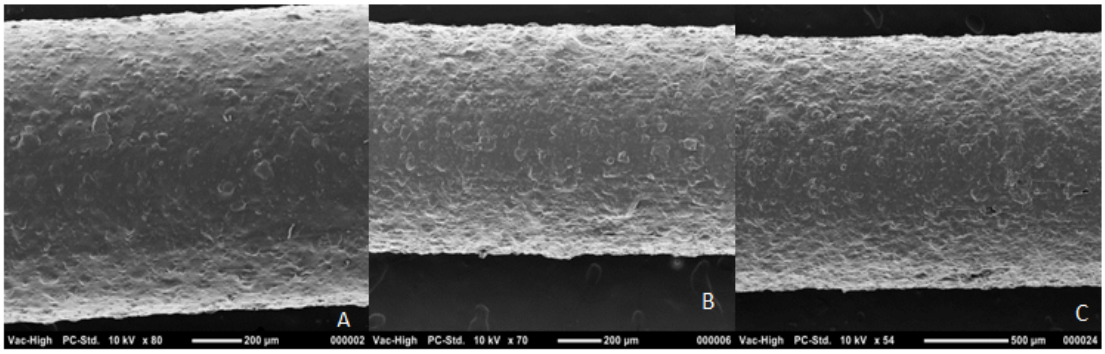

Figure 14. Scanning electron micrographs of implants with a ratio of (A) 1:10 (JA1424-01), (B) 2:10 (JA1424-02) and (C) 3:10 (JA1430-02) core mixture: polymer.

4.2.4. Effect of Mannitol

The presence of mannitol in the core mixture as a stabilizing agent has a huge effect on the surface morphology of the implant (Figure 15). Both formulations present in Figure 15 were prepared with protein stabilized by spray drying which produces small particle sizes. During the process of extrusion the mannitol particles remain dispersed in a solid state which leads to an irregular surface when compared with a formulation without mannitol.

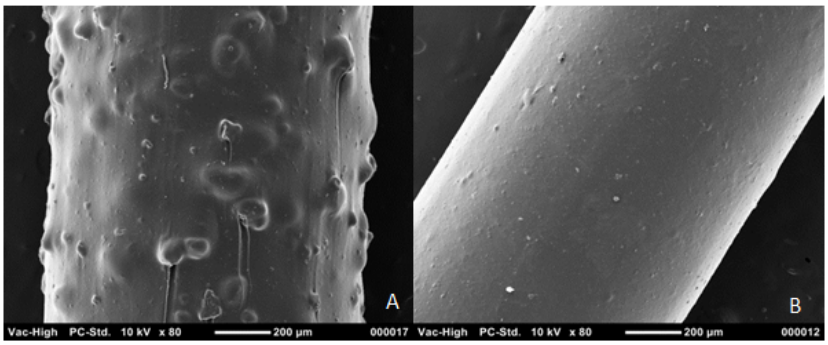

Figure 15. Scanning electron micrographs of implants (A) with mannitol (JA1416-01) and (B) without mannitol (JA1416-02) in the formulation.

4.3. *In vitro* release study of the implants

In order to understand the release mechanism of the extruded implants with different settings an in vitro release study was performed following the procedure present in the Materials and Methods section. The main objective of this work was the studying of the parameters that can influence the biphasic release kinetics of a hot melt extruded implant therefore, an *in vitro* release study is relevant.

4.3.1. Parameters influencing burst release

In Figure 16 is presented the influence of the implant drug loading on the burst release and the result shows that there is no big difference between the implants loaded with a ratio of 1:10 and 2:10 core mixture:polymer. Low loadings result in low burst and low *plateau* level, below 10%. On the other hand, the increase of drug loading results in a more sustained release kinetics and an increase of total protein released over the same period of time. When an implant loaded with high loading starts releasing the drug, even for small drug particles, more pores are formed in the implant structure. Furthermore, the presence of inulin as a lyoprotectant in the matrix of the polymeric device can help in the formation of pores. With higher loadings there are more particles, containing both drug and inulin, closer to each other which means that sugar particles can be connected. Once in contact with water sugar dissolves and pores are easily formed which can severely influence drug release profile. With this porous matrix it is easier for the drug to diffuse for the release medium. To find the desired release kinetics in terms of burst release other ratios between the core mixture and the polymer can be tested. Furthermore, the batch with a ratio of 2:10 should be repeated and consolidated as the tendency once increasing drug loading seems to be an incremental cumulative drug release which does not happen when comparing ratio 1:10 and 2:10.

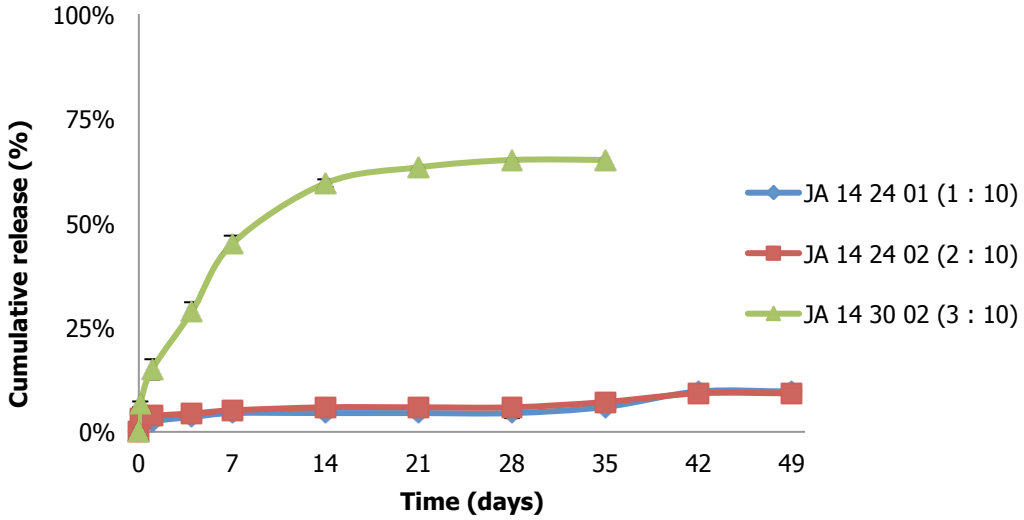

Figure 16. Schematic representation of release kinetics of different loaded implants.

The state of the protein used for the implants preparation has a huge difference on burst release (Figure 17). Using non-stabilized protein leads to a higher burst release and a higher *plateau* level when compared with the stabilized proteins. Burst release is often associated to the proteins present in the implant surface. For bigger particles, like the non-stabilized protein particles the burst must be higher because when the surface proteins diffuse to the medium big pores are formed that can be connected allowing the protein inside to diffuse for the release medium. The stabilized proteins have a smaller particle size therefore, the molecular diffusion is slower and the formed pores are smaller therefore, it is more difficult to have connected pores inside of the implants which would lead to a higher burst release.

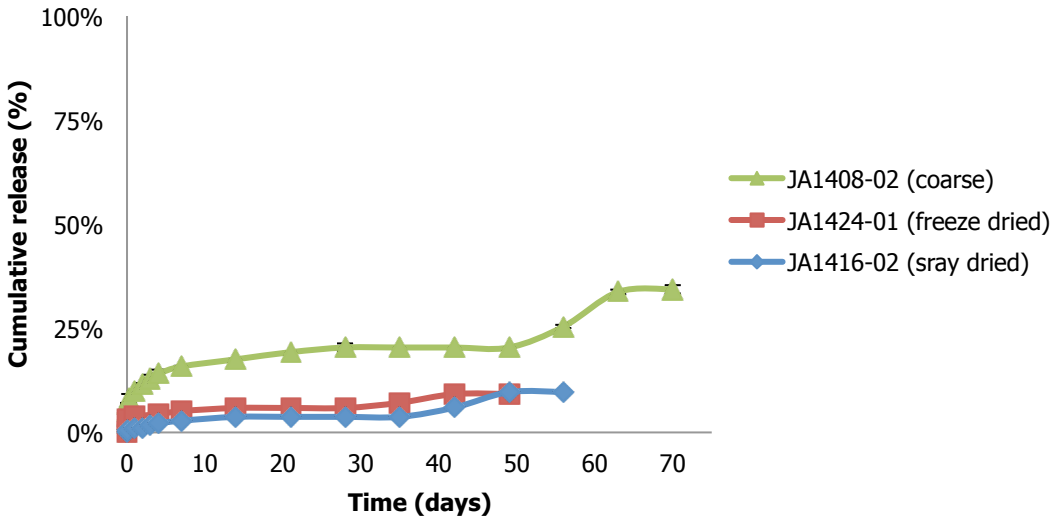

Figure 17. Schematic representation of release kinetics of implants made with different proteins.

Figure 18 presents *in vitro* release kinetics for different polymers. The formulations prepared with PDL 05 have a higher burst release when compared to the other formulations. This polymer has a *Tg* between 42-47 ºC thus, at release temperature (37ºC) the implants prepared with this polymer are below *Tg*, the molecular mobility is low and the pores in the formulation are open. On the other hand, the *Tg* of PDL 02 is between 32-37ºC thus, at release temperature they are above *Tg*, the material is in a rubbery state and the pores which could be present in the material will probably close.

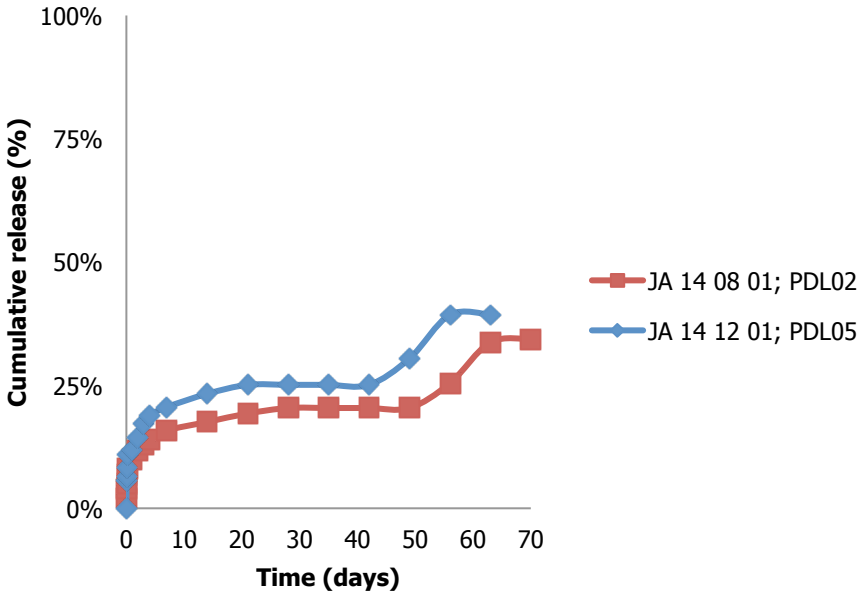

Figure 18. Schematic representation of IVR kinetics from implants with different materials.

4.3.2. Parameters influencing on set and slope of secondary pulse

Huge differences can be observed in the release behavior of PDL-based formulations when comparing to PDLG-based formulations (Figure 19). Since the drug release from the implants is degradation-dependent, polymer degradation kinetics play an important role on the drug release behavior. Co-polymerization of poly-lactic acid with glycolic acid reduces the crystallinity of the polymer and increases the ability of water to penetrate the matrix compared with pure poly-lactic acid. Therefore, such co-polymer gave rise to a faster degradation ratio compared to PDL-based materials when compared to a faster drug release which is generically obtained from the implants containing glycolic acid. Additionally, the presence of glycolic acid in the co-polymer leads to a higher accumulation of acidic products inside of the implant, which accelerates the polymer degradation even more and also justifies the faster drug release. This effect may also be responsible for the sharper pulse of the PDLG materials. With 20CP15C20-C40 we are expecting a low swelling degree thus, a slower degradation however, until now, is not possible to conclude anything from the release kinetics, except from a low burst release.

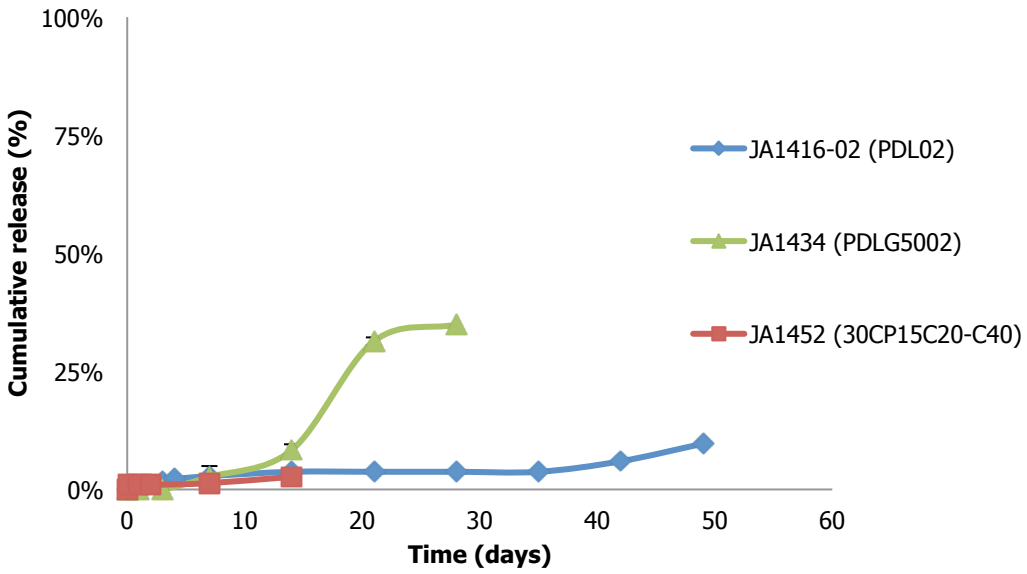

Figure 19. Schematic representation of IVR kinetics from implants prepared with different polymer grade.

There was a huge effect induced by the state of the protein used with the second pulse (Figure 17). When the polymer starts degrading more pores will be formed which allows the protein to diffuse for the outside of the device. As bigger and bigger particles leave the device throughout time, more and more pores are formed which induces easier connections between pre-existing pores and a consequent easier diffusion of the protein to the external environment. As it happens with burst release behavior, the diffusion of smaller particles will create smaller pores thus, it is more difficult to have connections between the pores.

Since the polymer degrades by hydrolysis, a slower degradation is expected for a larger implant diameter (22). In accordance, also slower drug release kinetics is expected from larger implants. Actually, implants with larger diameters offer a longer diffusion distance of the immobilized drug once the pore size has been increased by polymer degradation and allows drug release to the external environment (22). An identical rational is also valid for the entrance of water which will induce polymer degradation. In practice, from the present study there was no significant difference between 1.0 and 1.4 mm implants. However, a small difference is observed for the smaller implants (0.6 mm) when comparing to the larger ones, as they release the drug slightly faster, which is in accordance with theoretical analysis.

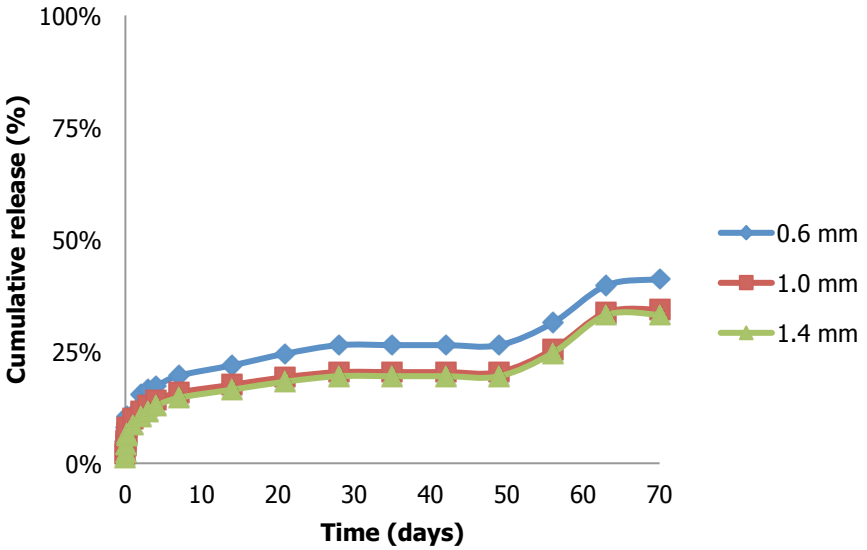

Figure 20. Schematic representation of IVR kinetics from JA1408 implants with different diameters.

The presence of mannitol has no effect on burst release however, a small effect is observed in the slope of the secondary pulse. Mannitol is a fast dissolving sugar thus, it was expected a higher release since the dissolution of the sugar will be responsible for the pore formation and the drug could diffuse for the outside of the device.

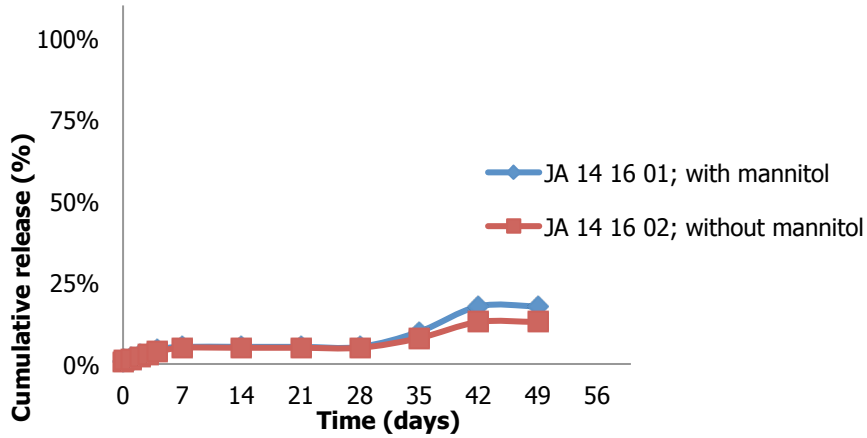

Figure 21. Schematic representation of IVR kinetics from implants with and without mannitol.

Hot melt extrusion is a process that allows the production of implants with a bi-phasic release. Changing the polymer from PDL 02 to PDL 05 leads to an increase in the burst release. PDL 02 based formulations loaded with spray dried or freeze dried inX shows a low burst release (less than 5%), hardly any release between burst and secondary release pulse, second release pulse starts after 35 days, although the slope of the pulse is not as sharp as initial requirements for a biphasic drug release. Using polymers with glycolide in the composition leads to a shorter lag phase and to an increase in the slope of the secondary pulse. Theoretically, larger implant diameters lead to a slower drug release however, the same logic was not clearly observed in the formulations prepared.

4.4. Incomplete protein X release - Discussion

From the release kinetic analysis it is possible to observe that after a second release pulse there is a *plateau level*. Therefore, is important to understand if the protein was lost during the release or if protein was still remaining inside of the implant. A root analysis was made to understand in which steps the protein was lost. For that, a flowchart (Figure 22) with the possible critical parameters was created and an analyse to those parameters was made.

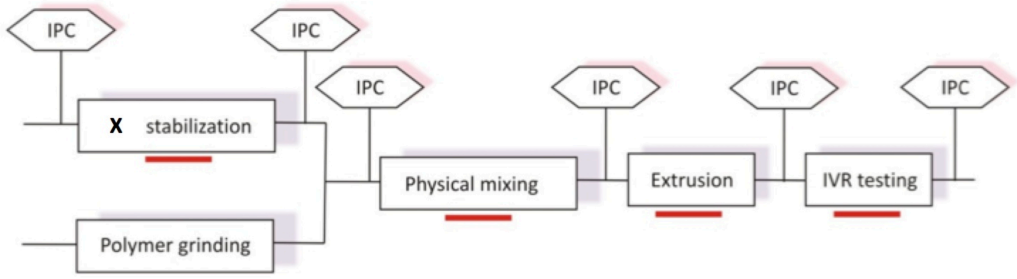

Figure 22. Flowchart of preparation of protein X loaded extrudates.

4.4.1. Protein X losses during manufacturing process

To perform a detailed characterization of the implants a content determination method is necessary to quantify the exact protein X loading. Additionally, such analysis also allows to the amount of protein lost during manufacturing process. Both parameters are represented in Table 15.

Table 15. protein X content of the implants after manufacturing process.

Batch ID	Polymer grade	Protein type	Extrusion temperature (°C)	Theoretical loading (wt.%)	Actual loading (wt.%)	Protein loss (%)
JA1408	PDL02	Coarse X	70	0.36	0.23	35
JA1410	PDL05	Coarse X	75	0.36	0.19	44
JA1416-01	PDL02	inX (SD)	70	0.0585	0.581	0.6
JA1416-02	PDL 02	inX (SD)	70	0.81	0.61	24
JA1424-01	PDL02	inX (FD)	70	0.82	0.75	8
JA1424-02	PDL02	inX (FD)	70	1.47	0.85	42
JA1430-02	PDL02	inX (FD)	70	2.08	1.90	8
JA1434	PDLG5002	inX (SD)	50	0.84	0.61	27
JA14523	30CP15C20-C40	inX (FD)	47	0.29	0.22	23

During the manufacturing process protein losses from 8% to 44% were detected and the variation inter-batch is very significant even for similar batches. Thus protein losses cannot be easily associated with specific process parameters or materials used. Although, in general

terms, batches using non-stabilized protein (coarse X) gave rise to higher protein losses when comparing to batches using stabilized proteins, which clarifies the utility of inulin. After the analysis of these results, the similar behavior of JA1424 implants is justified by the similar loading of both.

Several parameters can influence the loss or denaturation of protein during the manufacturing process: the protein stabilization with inulin, the physical mixing in the mortar, the temperature and the shear stress during extrusion. In order to understand and identify the critical steps which mainly influence protein stability all of the parameters were studied excepting the effect of shear stress since it would represent a prohibitive spending of protein due to the minimum loading amounts on the extruder equipment in use.

The exact loading of the stabilized protein in inulin is represented in Table 16. According to the data collected, independently of the batch, the difference between the theoretical loading and the real loading is not significant (<2%), thus, we assume that the stabilization process is not detrimental for the protein.

Table 16. Determination of real content of stabilized protein X.

Batch number	Protein State	Theoretical loading (wt%)	Exact loading (wt%)	Protein loss (wt%)
NG14B03A	SD	8.44	8.48	< 1%
JA1451	FD	9.49	9.34	1.4%

In order to evaluate the effect of polymer, protein and inulin mixture using a mortar, all the components were mixed for 10 minutes and protein was quantified via UPLC to detect the percentage of protein losses in the process.

Table 17. Determination of protein X loss during physical mixing.

Batch number	Theoretical loading (based on in-weights)	Exact loading (content determination)	Protein loss (wt%)
JA1458-02	0.35 wt.%	0.31 wt.%	13%

According to Table 17 more than 10% of protein is lost during the physical mixing, which can be caused by the formation of aggregates or adsorption to mortar. Changing the porcelain mortar to an agate mortar which has a smoother surface could be a good alternative approach to overcome the protein losses during physical mixture.

To verify the effect of the temperature in protein stability, the protein was exposed to 50^{o}C and 75^{o}C during 5, 10 and 15 minutes, these times represent an approximation to the residence time of the protein inside of the extruder. And the temperatures were chosen taking into consideration maximum and minimum temperatures used during extrusion process.

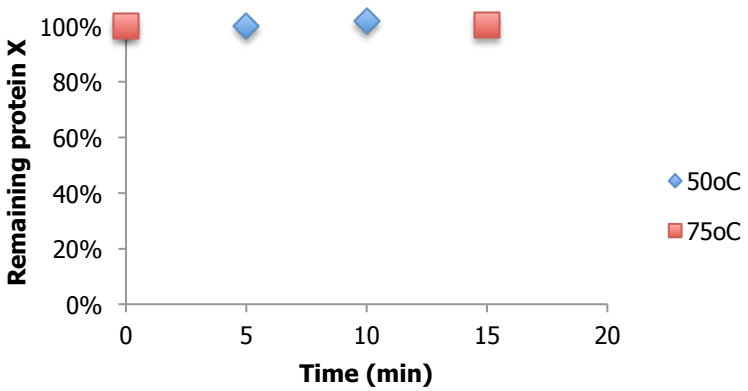

Figure 23. Schematic representation of remaining non-stabilized protein X after heating exposure.

Figure 23 shows the amount of remaining protein after 5, 10 and 15 minutes at 50ºC or 75ºC. As the protein is stable at these temperatures, the temperature effect seems not to be a detrimental factor for the protein loss or denaturation during extrusion. Therefore, we conclude that the shear forces inside of the extruder and the physical mixing step may represent the main causes for protein loss during the extrusion.

4.4.2. Protein X losses during *in vitro* release

From the release kinetic analysis it is possible to observe that after a second release pulse there is a *plateau* level. Therefore, in order to understand if the protein was lost during the release or if protein was still remaining inside of the implant, a determination of the drug content in the implants was made. The results are present in Table 18.

Table 18. Remaining protein X inside of the implants.

Extrusion run	Polymer grade	State of protein	Loss during release	Remaining protein X (wt.%)
JA1408	PDL02	Coarse	30 %	< LOD
JA1410	PDL05	Coarse	20 %	< LOD
JA1416-01	PDL02	inX (SD)	65 %	< LOD
JA1416-02	PDL02	inX (SD)	86%	< LOD
JA1424-01	PDL02	inX (FD)	80 %	< LOD
JA1424-02	PDL02	inX (FD)	50 %	< LOD
JA1430-02	PDL02	inX (FD)	27 %	< LOD
JA1434	PDLG 5002	inX (SD)	40 %	< LOD

As no remaining protein X was found inside of the implants after content determination we hypothize that an irreversible interaction between protein X and the polymer and/or an interaction between the protein and polymer degradation products may be occurring.

4.4.2.1. Interaction between protein X and the polymer.

To determine if the contact between the protein and the polymer is detrimental to the protein a solution with a target concentration of protein was incubated with 3 different polymers: PDL 02, PDLG 5002 and Poly-ε-caprolactone for four days at 37ºC and the decrease in protein concentration was analyzed. The maximum protein loss was 5% so we can assume that the interaction between the protein and the polymer is not a main factor for the protein loss during in vitro release.

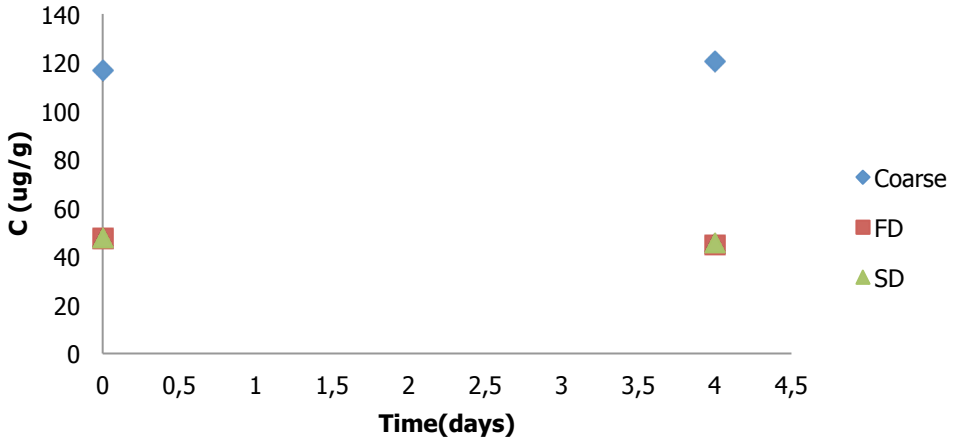

Figure 24. Protein X concentration after four days incubated with PDL 02.

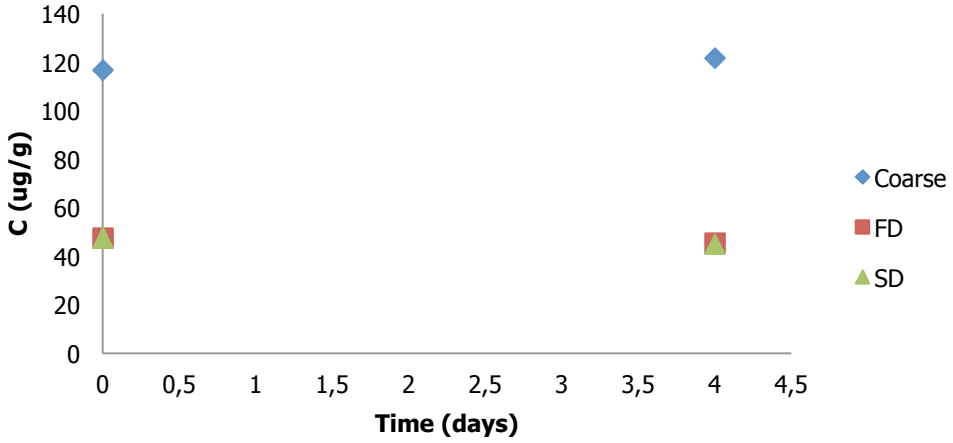

Figure 25. Protein X concentration after four days incubated with PDLG 5002.

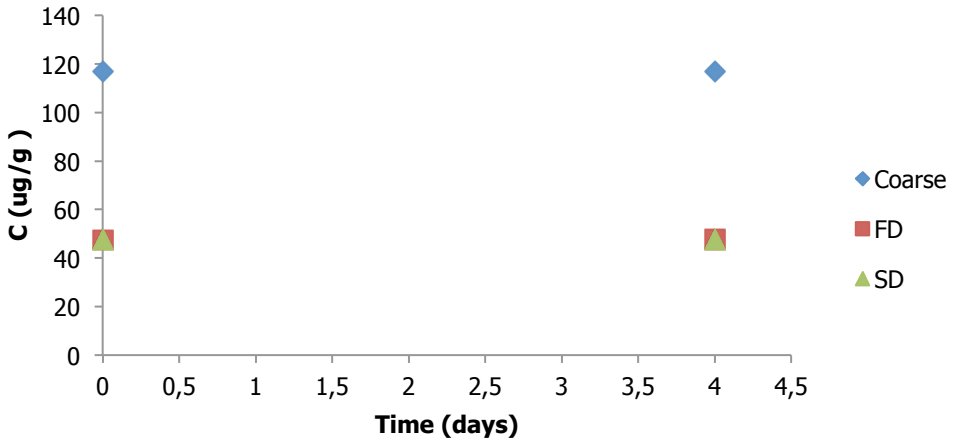

Figure 26. Protein X concentration after four days incubated with Poly- ε -caprolactone.

4.4.2.1. Interaction between protein X and product degradation products

To analyze the effect of degradation products in the stability of protein X, a protein solution was mixed with 2000 μg/g of a monomer solution containing D,L Lactide, Glycolide and **ε** -Caprolactone. The mixture was then incubated at 37ºC for 14 days and the decrease of intact protein X concentration was analyzed. For non-stabilized protein incubated with D,L Lactide and glycolide solutions approximately 25% of the protein was lost while under similar conditions following incubation with **ε** -caprolactone just about 10% was lost. Such result may have occurred due to the more acidic climate created in the solutions composed by D,L lactide and glycolide monomers since the pH was close to 2 while for the **ε** -caprolactone solution was about 3. For stabilized protein by freeze drying, 25% of protein was lost when was incubated with a D,L Lactide solution. For **ε** -caprolactone and glycolide solutions only 10% of the protein was lost. The incubation of the stabilized protein by spray drying in **ε** -caprolactone and D,L Lactide led to a loss of 25% of the protein while for the incubation with glycolide only 12% are lost. Based on the results obtained, we hypothesize that the stabilization of the protein using inulin is not effective in liquid state since the sugar will quickly dissolve in the medium.

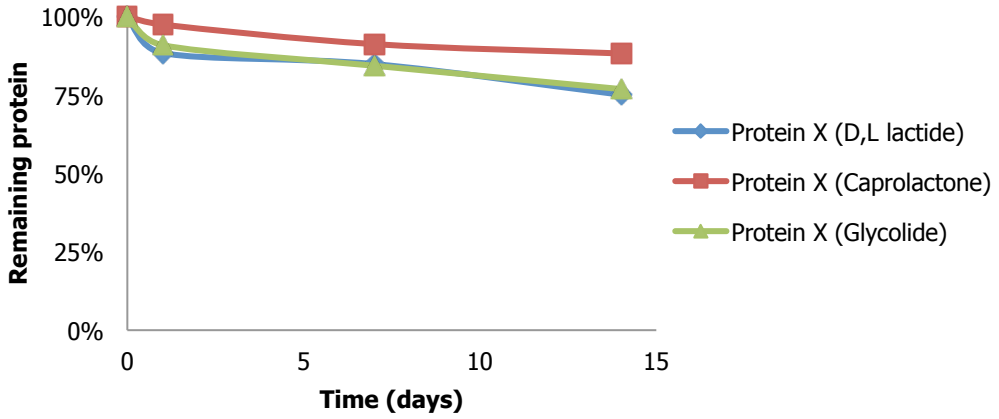

Figure 27. Schematic representation of the amount of remaining coarse protein after incubation with monomers solutions.

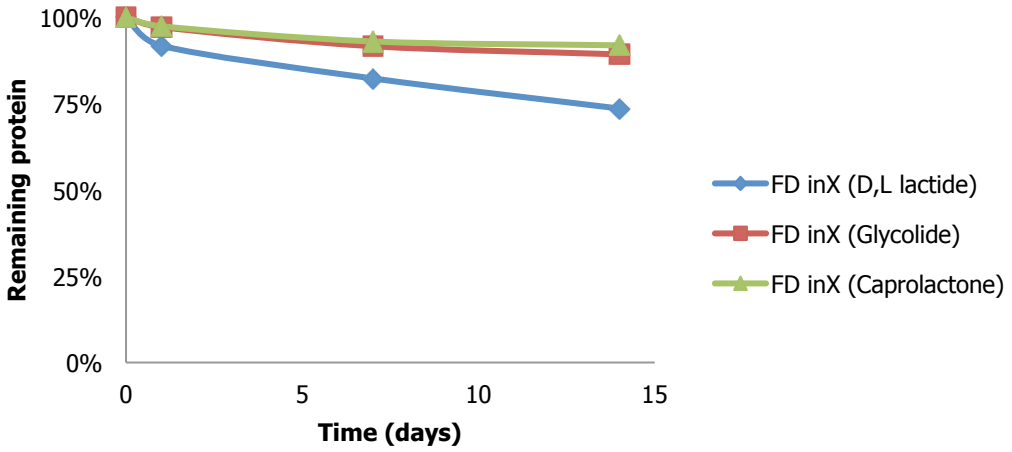

Figure 28. Schematic representation of the amount of remaining FD protein after incubation with monomers solutions.

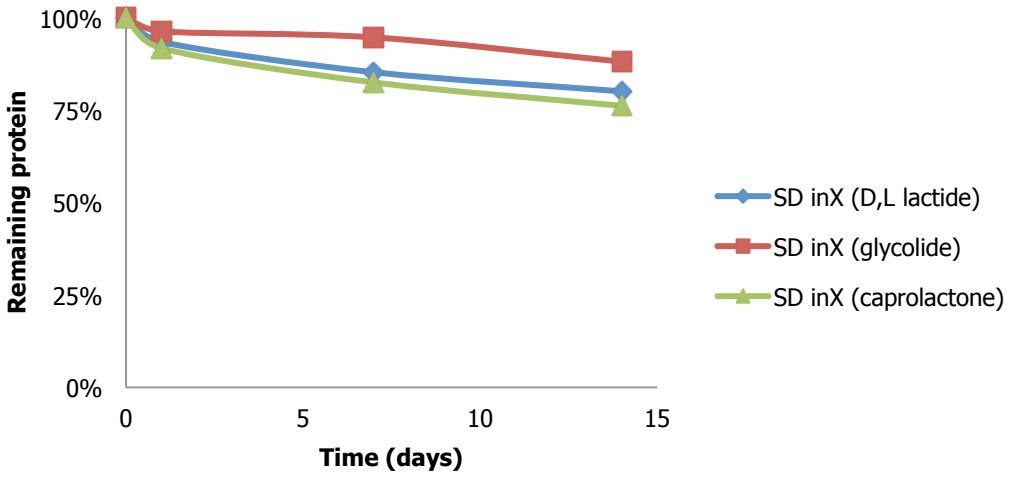

Figure 29.. Schematic representation of the amount of remaining SD protein after incubation with monomers solutions.

In order to confirm whereas the protein is stable when exposed to low pH levels, three independent solutions of 20% wt of a single monomer (lactide, glycolide, **ε** -caprolactone) were prepared (monomers were hydrolyzed overnight at 50°C) to study their effect on the protein which was then added on the solid state. The three solutions represent acidic degradation products and before protein addition (target concentration of 200 μg/g) the pH of every solution was measured and adjusted to 3 using NaOH, as this pH level is close to the expected inside of implants under bulk degradation. When the protein was added, no dissolution was observed, probably as a result of the acidic climate which may have caused protein denaturated and formation of aggregates as Figure 30 illustrates. As the pH of the solution was close to the isoelectric point of the protein, besides the possible denaturation the dissolution of the protein also became more difficult. The isoelectric point is the pH of a solution at which the primary charge of a protein becomes zero. If the pH of the solution is above the isoelectric point the surface of the protein is negatively charged consequently, repulsive forces are predominant. On the other hand, if the solution pH is below the isoelectric point the protein surface is positively charged and repulsion between molecules will also occur. However, when the solution has a pH close to the isoelectric point of the protein, positive and negative charges will cancel and, therefore, repulsive electrostatic forces are reduced and the attraction forces become more predominant. Ultimately, such attraction forces may cause aggregation and precipitation as detected during the current experiment.

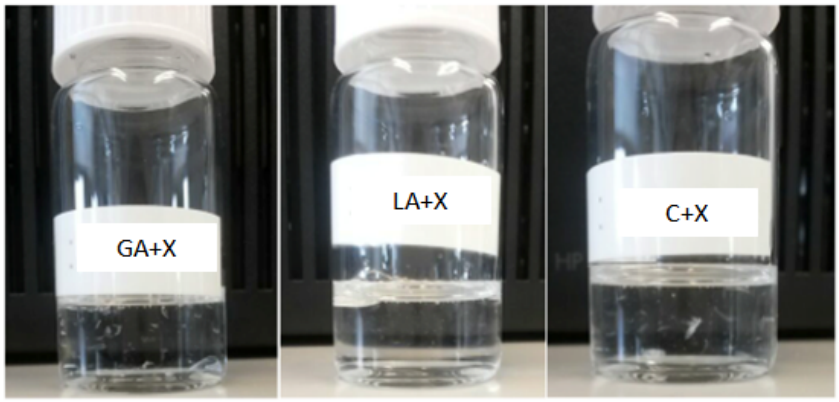

Figure 30. Visual aspect of monomer solutions with protein X.

At low pH the protein takes on a molten globular for min which flexibility of the side chain is increased decreasing stability and changes on the tertiary structure are observed (27).

For all states of protein incubated with D,L Lactide and **ε** -caprolactone solutions about 98% of active protein was lost while after incubation with glycolide about 70% of protein in an active state was lost (Figure 32, Figure 33 and Figure 34). This experiment was a simulation of the behavior of the protein when in contact with degradation products inside of implants. Thus, the decreasing in the protein content is more evident than when the protein is

incubated with a solution with low concentration of monomers. This experiment justifies the incomplete release of the protein from the implants after the lag phase. In Table 18, it is possible to observe that from the batches with stabilized protein and with a ratio of 1:10 core mixture: polymer (JA1424-01, JA1424-02 and JA1434) the losses during the release are different. A small amount of protein is lost in formulations with PDLG when compared with PDL formulations. These results are in accordance with the results obtained by this experiment since the incubation with glycolide monomers has led to small losses during in vitro release.

In the chromatogram it was possible to observe an additional peak which can be associated to the degraded protein. Furthermore, this peak tends to appear in the last measurements of the samples undergoing *in vitro* release studies which demonstrate an increasing susceptibility of the protein X to acidic environments.

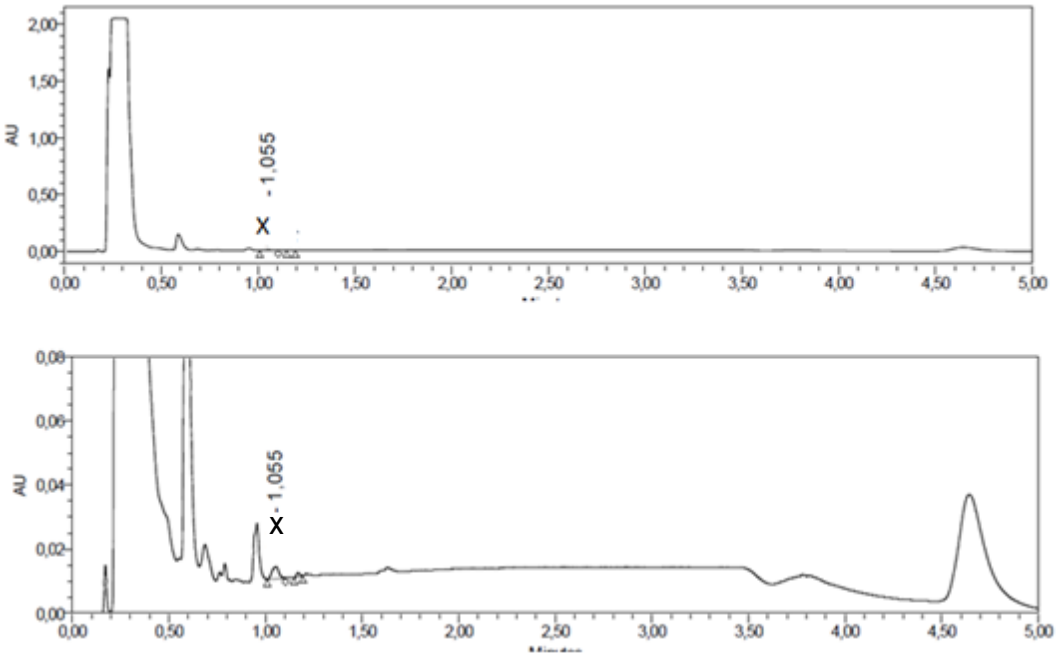

Figure 31. Chromatogram obtained for protein X incubated with Glycolide for 4 days.

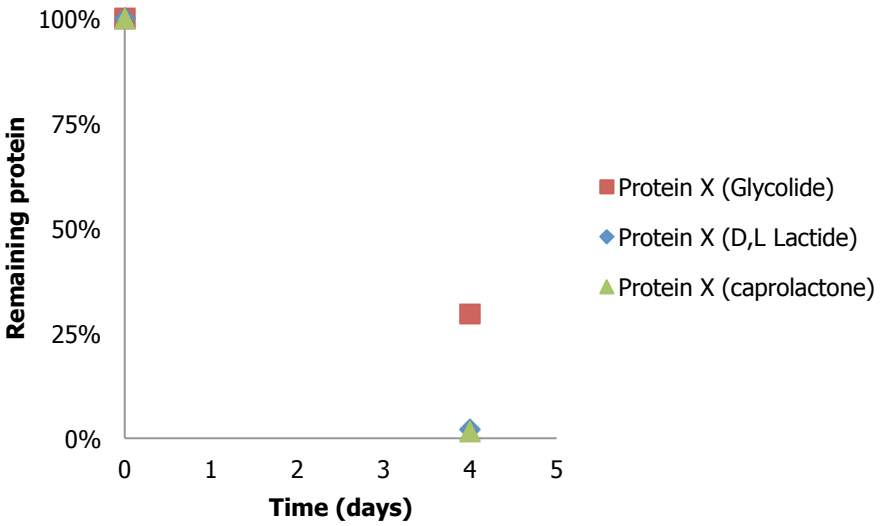

Figure 32. Schematic representation of the remaining amount of protein X after incubation with a 20%wt monomer solution.

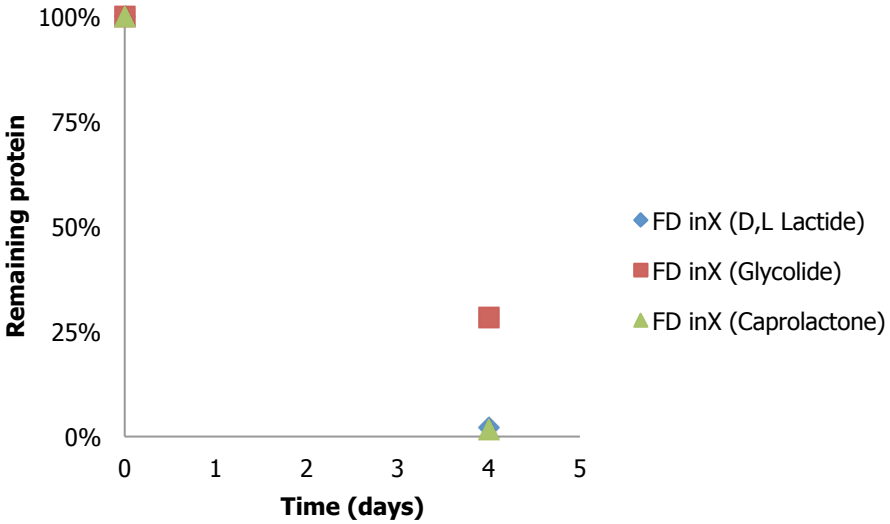

Figure 33. Schematic representation of the remaining amount of FD protein X after incubation with a 20%wt monomer solution.

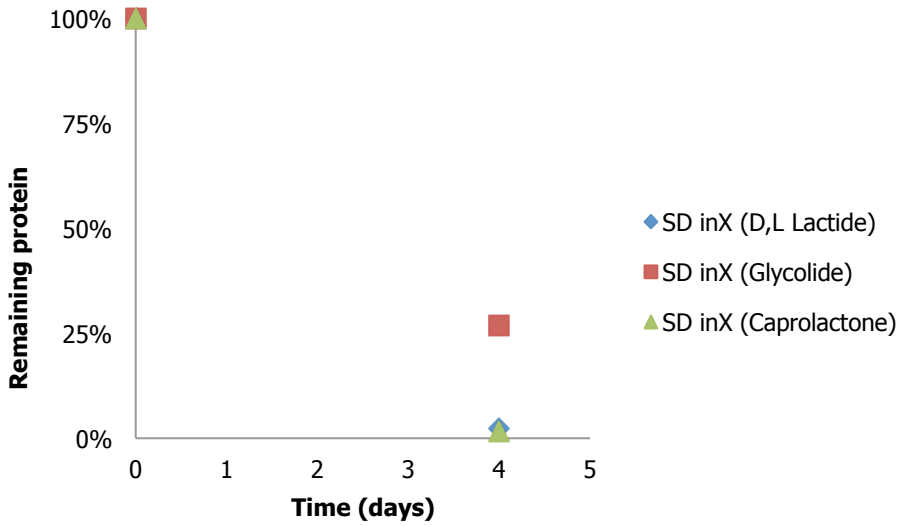

Figure 34. Schematic representation of the remaining amount of FD protein X after incubation with a 20%wt monomer solution.

Table 15 illustrates the variability of lost protein during *in vitro* release studies from batch to batch. The extrusion process requires higher temperatures to melt the polymer. After extrusion, the implant is a dense structure, consequently, during release it is more difficult the diffusion of the acid degraded products (22). The batches with smaller protein losses are the ones prepared with non-stabilized protein. As referred previously, with the diffusion of the protein out of the devices a porous structure is formed inside of the implant, which facilitates the diffusion of another drug molecules as well, the degradation products. On the other hand, for stabilized proteins with a low loading (JA1416, JA1429-01 and JA1429-02) the pores formed by the protein diffusion are not enough to allow the diffusion of the degradation products thus, they stay inside of the implant and it is formed an acidic microclimate which is detrimental for the protein. In the case of an implant with an higher loading like JA1430-02 the mechanism is similar to the ones with non-stabilized protein, with the diffusion of the drug the structure became more porous and the diffusion of the degradation products is facilitated.

JA1434 implants were prepared with PDLG, which is a faster degrading polymer due to the glycolide content thus a porous structure is easily formed when compared with PDL materials. However, the presence of glycolic acid in the medium is more detrimental to the protein (27). This effect is counteracted by the faster release of the drug thus, compared with the formulations made with PDL less protein was lost.

5. Conclusions

The objective of this work was to produce implants as a drug delivery system and to study the effect of several process parameters such as drug loading, polymer composition and the state of the protein to be released from implants.

Hot melt extrusion is a process that allows the production of implants with a bi-phasic release. Changing the polymer from PDL 02 to PDL 05 leads to an increase in the burst release while using polymers with glycolide in the composition (PDLG-based co-polymers) leads to a shorter lag phase. The state of the protein also influences the release kinetics, for non-stabilized protein the burst release is higher when compared with stabilized proteins. Theoretically, larger implant diameters lead to a slower drug release however, the same logic was not clearly observed in the formulations prepared.

The accumulation of acidic degradation products inside of the implant is crucial to obtain a bi-phasic drug release. For this study, the protein used denatured during release, the accumulation of degradation products inside of the implant leaded to an acidic micro-environment which is detrimental for the protein. At that low pH the protein loses the native conformation leading to a loss in the activity.Therefore, drugs which are highly sensitive to acidic environments seem not to be suitable for such therapeutic approach.

6. Future work and recommendations

In order to further study and understand the mechanisms influencing bi-phasic release of a drug, an API stable at low pH should be used instead of protein X.

During the manufacturing preparation, the porcelain mortar should be replaced for an agate mortar since the last one has a smother surface. This modification is expected to enhance mass yields, although after this replacement a batch of implants should be made to analyze the exact losses due to the shear forces applied.

In incorporation of magnesium hydroxide in the formulation could help raising the acidic microclimate inside of the device allowing this type of devices to be a pH sensitive drug carrier.

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